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# Cultivation of *Chlorella Sorokiniana* Using Beef Feedlot Runoff Holding Pond Effluent

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CULTIVATION OF *CHLORELLA SOROKINIANA* USING  
BEEF FEEDLOT RUNOFF HOLDING POND EFFLUENT

by

Mitchell Klaus Goedeken

A THESIS

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Amy Millmier Schmidt and Yufeng Ge

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Cultivation of *Chlorella Sorokiniana* Using  
Beef Feedlot Runoff Holding Pond Effluent

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University of Nebraska, 2017

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A study was conducted to test the suitability of utilizing beef feedlot runoff holding pond effluent for cultivating algae. The algae strain used, *Chlorella sorokiniana*, was previously identified as a potential energy feedstock for cattle. The previous research was initiated in pursuit of a goal to develop a cycle of utilizing nutrients from beef manure to cultivate algae and then utilizing dewatered algae as a feed supplement for beef cattle.

Runoff holding pond effluent samples were collected from commercial beef production operations in Nebraska during spring 2016. Equal portions of samples from each cooperating farm were composited and then aliquoted into vessels to which treatments were randomly applied. Treatments were designed to evaluate algae growth under varied dilutions of effluent, pre-treatment processes, and supplementation with nitrogen and phosphorus. Growth characteristics under treatments were compared to algae growth in Bold's Basal Medium (BBM).

Algae concentration under treatments was evaluated daily by manual enumeration using a hemocytometer and via light absorbance using a spectrophotometer. A prediction

equation was then developed to assess the effectiveness of using light absorbance as a rapid method for quantifying cell density in runoff holding pond effluent.

Only one treatment, 60% autoclaved pond effluent diluted with water, was effective for cultivating algae to a concentration similar ( $p < 0.05$ ) to the BBM treatment. The prediction model was reliable ( $R^2=0.75$ ) for samples with algae concentrations greater than 200,000 cells mL<sup>-1</sup>.

Study results suggest that the nutrient profile of beef feedlot runoff holding pond effluent is suitable for growing *Chlorella sorokiniana*, but competition for nutrients or consumption by other organisms inhibits growth. While the prediction of algae cell concentration in solutions containing beef feedlot runoff holding pond effluent using light absorbance appears to be valid at algae cell concentrations above 200,000 cells mL<sup>-1</sup>, suspended solids in effluent, the presence of non-algae microorganisms, and low algae cell density at the initiation of the trials limits the ability of the model to accurately predict cell counts under these conditions.

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## CHAPTER 1: REVIEW OF LITERATURE

### 1.1 Introduction

With 6.45 million head of cattle, 38% of which are maintained in feedlots (USDA-NASS, 2016), Nebraska accounts for seven percent of the United States cattle inventory. Beef cattle can excrete up to 65 pounds of manure per day per 1000 pounds of live weight (USDA-NRCS, 2008). Regardless of the size of a feedlot operation, the manure generated by the confined cattle must be managed to avoid discharges of pollutants to waters of the state.

Management of manure from beef feedlots typically involves two independent waste streams: solid material accumulated on the feedlot surface and manure-laden stormwater runoff. Feedlot surfaces are comprised of two layers: the exposed surface containing loose manure pack and a consolidated layer of manure and soil beneath the surface material (Woodbury et al., 2001). The loose manure pack is commonly scraped at least once per year and stockpiled until conditions are favorable for application of this material to cropland as a fertilizer. Stormwater that comes into contact with feedlot surfaces, either from direct deposition during precipitation events or from overland flow entering the lot, must also be managed to prevent unintended discharges to surface waters. Precipitation and snowmelt in sufficient quantities to cause runoff will carry dissolved solids and nutrients from the feedlot surface. Surface runoff is commonly allowed to gravity-flow to a storage structure where the liquid is held until application to cropland is possible. The stored liquid, known as runoff holding pond effluent, is typically very dilute with approximately 0.3% solids content (Fulhage et al., 2001). As

with the stockpiled manure solids, runoff holding pond effluent is applied to cropland to provide plant nutrients when conditions are favorable.

### **1.2 Runoff Holding Pond Effluent Characteristics**

The nutrient composition of feedlot runoff holding pond effluent can vary considerably among operations due to management practices, diet composition and digestibility, geographic location and seasonality (Dickey and Vanderholm, 1977). Rainfall frequency and intensity impact effluent nutrient characteristics significantly (DeLaune et al., 2012). Concentrations of total nitrogen (TKN), ammoniacal nitrogen ( $\text{NH}_3\text{-N}$ ), phosphorus as phosphate ( $\text{P}_2\text{O}_5$ ), and potassium as potash ( $\text{K}_2\text{O}$ ) in runoff holding pond effluent, while variable, average 0.20, 0.18, 0.00 and 0.90 kg L<sup>-1</sup>, respectively (USDA-NRCS, 2008; Fulhage et al., 2001). Because of the relatively dilute nature of holding pond effluent, its value as a fertilizer is minimal. However, discharge of the effluent is disallowed under the Clean Water Act, so the dilute liquid is irrigated onto a growing crop to utilize the nutrients. Utilizing the dilute nutrients in the effluent for value-added production of algae prior to cropland irrigation would exclude little nutrients from crop production application and could provide a profitable addition to the beef production operation.

### **1.3 Value of Algae**

Algaculture, the large-scale production and cultivation of algae for commercial and industrial uses, has existed for more than 60 years in the United States and other countries (Borowitzka, 2013; Tamiya, 1957). The cultivation of microalgae in many geographic regions of the world demonstrates the evolution and adaptation of microalgae to environments worldwide (Fehling et al., 2007).

Algal biomass has been shown to contain nitrogen in concentrations comparable to fertilizers made from composted manure and algal biomass can be used as a slow-release fertilizer (Wilkie and Mulbry, 2002; Oswald, 1988). Wilkie and Mulbry (2002) showed that algal biomass grown using anaerobically digested dairy manure could contain approximately 2.8% nitrogen if dewatered to 40% total solids. Nutrients taken up by algae may allow for greater control of nutrient application through distribution of the algal biomass (Oswald, 1988).

Algae have also been used as a source of nutritional supplementation in humans for thousands of years (Kiple and Ornelas, 2000), primarily as sources of lipids, omega-3 fatty acids, metal supplements, and antioxidants. Because nutrient concentrations can vary considerably among algae species, the selection of a species for any particular nutritional application should take into consideration the intended use (Wells et al., 2016). Some algae readily adsorb heavy metals, making nutrient toxicity a concern when algae are used as a dietary supplement (Gadd, 2008; Turner et al., 2009); therefore, it is important to understand the chemical characteristics of the feedstock during cultivation of algae for human or animal consumption (Wells et al., 2016).

The production and cultivation of algae using municipal wastewater as a feedstock has been investigated as a tertiary water treatment process (Li et al., 2011; Hernandez et al., 2006; Abdel-Raouf et al., 2012; Lizzul et al., 2014). Li et al. (2011) demonstrated that centrate produced during the activated sludge thickening process in a wastewater treatment system could be utilized as an algae feedstock. In this study, *Chlorella sp.* was used to reduce ammonia concentration by 93.9%, total nitrogen by 89.1%, total phosphorus by 80.9%, and carbon oxygen demand by 90.8%, lessening the

need for tertiary treatment of the wastewater stream and producing a valuable by-product. Yun et al. (1997) succeeded in using *Chlorella vulgaris* to capture CO<sub>2</sub> at a rate of 26.0 g-m<sup>-3</sup>-h<sup>-1</sup> and remove ammonia at a rate of 0.92 g-m<sup>-3</sup>-h<sup>-1</sup> from raw wastewater discharges.

Biofuels derived from the cultivation of algae provide another value-added opportunity to utilize nutrients in wastewater streams while generating a product capable of off-setting reliance on petroleum-based fuel products (Bibi et al., 2017; Park et al., 2011; Pittman et al., 2011; Lin and Lin, 2011). There are two main ways to generate biofuels from algal biomass. The algal biomass can be fermented and the alcohol generated during this process can be collected and refined to ethanol (Bibi et al., 2017). Another method involves extracting and refining the oils in algae, which typically accumulate as saturated fatty acids, polyunsaturated fatty acids, glycolipids, or triacylglycerols (Pittman et al., 2011). These oils can replace many petroleum based products that are currently relied upon, including ethanol, diesel, lubricants, and others (Bibi et al., 2017). Bioplastics can be produced similarly to biofuels from algal biomass (Hempel et al., 2011; Zeller et al., 2013). Hempel et al. (2011) created a symbiotic relationship between poly-3-hydroxybutyrate producing bacteria and microalgae species *Phaeodactylum tricornutum*, where the poly-3-hydroxybutyrate can be used as a building block for bioplastic. Zeller et al. (2013) used *Chlorella vulgaris* and *Spirulina platensis* biomass under a 24-ton press to create a bioplastic through a thermoplastic molding process.

While a number of applications have been investigated for developing value-added uses for algae, the cost-benefit economics are an important consideration.

Benemann (2008) reported that the nutrient and energy requirements for centrifugation and refining of algae often render algaculture cost-prohibitive. Traditional growth nutrients, energy to aerate or circulate ponds, temperature, maintenance of the growth media, and other mechanical requirements are also costly. Co-location of algae production with existing nutrient-rich waste streams alleviates the cost of nutrients and can be a reliable and feasible means of algae production (Pittman et al., 2011; Singh et al., 2010). According to Park et al. (2011), “wastewater treatment HRAPs [high rate algae ponds] are presently the only economically viable way to produce algal biomass for conversion to biofuels with minimum environmental impact.”

While algae growth is used to remove or utilize nutrients from waste streams, it has also been shown to benefit waste streams in other ways. Moawad (1968) reported that conditions that promote algal growth inhibit the survivability of coliform bacteria. Colak and Kaya (1988) showed that algae as a treatment for waste water can reduce chemical oxygen demand (COD) and biological oxygen demand (BOD) in industrial wastewaters. Algae also can be used to sequester heavy metals (Wells et al., 2016; Gekeler et al., 1988).

### **1.3.1 Methods for Growing Algae**

Multiple methods exist for propagating algae. High rate algal ponds (HRAP) are a method used to grow algae in large quantities on a commercial scale (Park et al., 2011). These large ponds are shaped like a racetrack and a paddlewheel or pump is used to circulate the algae at a constant rate (García et al., 2006; Park et al., 2011; Arbib et al., 2017). Pumps can be used to introduce carbon dioxide (CO<sub>2</sub>) into the ponds to create a more ideal environment for algae growth (Park et al., 2011; Arbib et al., 2017).

Photobioreactor is a broad term used to describe various methods of growing algae while controlling gas interaction, light interaction, and nutrients. Marcilhac et al. (2014) grew algae in a sealed cylinder photobioreactor that maintained constant temperature, agitation, and gas interactions to grow *Scenedesmus sp.* from combinations of anaerobically digested agriculture waste from swine and cattle operations. Lizzul et al. (2014) used a specially designed photobioreactor to control and collect gas entering and exiting an algae growth vessel to measure algal biomass yield from flue gases. Kobayashi et al. (2013) used hanging bags bubbled with compressed air to grow *Chlorella sorokiniana* from anaerobically digested beef manure. These are just three examples of photobioreactors built for the sole purpose of maintaining and controlling the growth conditions for algae.

### **1.3.2 *Chlorella sorokiniana***

*Chlorella sorokiniana* is a single-cellular alga that typically ranges in size from 3 to 4  $\mu\text{m}$  (Bohutskyi et al., 2016), though cells as small as 2  $\mu\text{m}$  and as large as 4.5  $\mu\text{m}$  have been reported (Shihira and Krauss, 1965; Lizzul et al., 2014). Maximum growth rates for *Chlorella sorokiniana* vary based on the growth medium and conditions. In controlled growth media under phototrophic conditions, the algae concentration can double in as little as four to six hours (Lizzul et al., 2014). Under mixotrophic conditions, which involve combining multiple energy sources, the addition of glucose to the medium can support a doubling in algae concentration in no more than three hours (Shihira and Krauss, 1965). *Chlorella sorokiniana* grows best between 35 and 40°Celsius (de-Bashan et al., 2008).

#### 1.4 Algae Cultivation in Livestock Waste Streams

Studies have demonstrated the potential suitability of anaerobic digestion effluent as a growth media for algae (Balsam and Ryan, 2003; Kobayashi et al., 2013; Marcilhac et al., 2014). Algae consume and retain manure nutrients and can be used as a bioenergy source (Bohutskyi and Bouwer, 2013). Several studies have concluded that the limiting factor when selecting a growth media for algae is the presence of nitrogen compounds (Lizzul et al., 2014). A large portion of nitrogen in raw manure is in the organic form, which is not readily available to plants. The anaerobic digestion process allows for the degradation and mineralization of organic nitrogen to ammonium nitrogen ( $\text{NH}_4\text{-N}$ ), yielding digestate with a greater proportion of plant available nitrogen (Field et al., 1984).

While research into cultivation of algae from livestock manure is relatively limited, key studies in recent years have demonstrated successful cultivation of algal cultures in substrates containing a variety of livestock and poultry manures. Singh et al. (2011) successfully cultivated *Chlorella spirulina* using poultry waste anaerobic digester effluent. Fermented swine manure supplemented with chemical additives was used by Hu et al. (2012) to successfully cultivate *Chlorella sp.* Kobayashi et al. (2013) evaluated three strains of *Chlorella sorokiniana* in media containing 10% anaerobically digested beef manure using hanging bags bubbled with carbon dioxide. They concluded that *Chlorella sorokiniana* UTEX 1230 produced a similar quantity of biomass to another *Chlorella sorokiniana* strain over 24-day trials. Due to greater concentrations of proteins and carbohydrates, and relatively low concentrations of lipids, UTEX 1230 has a greater potential as a cattle feedstock. Hu et al. (2012) also concluded that *Chlorella sorokiniana* has high potential as a feedstock for animals.



Proteins and carbohydrates are necessary energy requirements in cattle diets (NASEM, 2016). Kobayashi et al. (2013) reported maximum protein levels for *Chlorella sorokiniana* grown in BBM of 50 to 60% ash-free dry weight and 40% ash-free dry weight when grown in 10% anaerobically digested beef manure. Guccione et al. (2014) demonstrated that *Chlorella* strains could produce 40% dry weight of protein and under nutrient starvation could increase carbohydrate concentration to 50% dry weight. Kobayashi et al. (2013) reported maximum starch concentrations of 10 to 15% ash-free dry weight in algae produced using BBM and 20 to 25% ash-free dry weight in algae produced using anaerobically digested beef manure. These variations in starch concentrations may be due to nutrient content, but may also be due to the different strains of *Chlorella* used. Corn has a typical protein content of  $8.79 \pm 0.97\%$  and a starch content of  $72.07 \pm 3.18\%$ , both on a dry basis (NASEM, 2016). Comparing corn to *Chlorella sorokiniana*, protein concentration is much greater in the algae, but the starch content is lower. In many feeding scenarios, especially for growing cattle, supplemental feed that is high in protein and low in starch is ideal. Overall, *Chlorella sorokiniana* has potential for a feed supplement for beef cattle.

For the research presented in this thesis, initial trials were conducted to observe *C. sorokiniana* growth in anaerobically digested beef manure while attempting to mimic open pond design and limit gas interaction to only the top surface of the samples. The initial lab trials were designed to test a scaled-down version of a facility being built at the University of Nebraska East Central Research and Extension Center to test a concept of co-location of algaculture on a beef feedlot using anaerobically digested beef manure. The goal of the algaculture was to be utilized as a beef feed additive. Dilution of 10%

anaerobically digested beef manure mixed with purified water ( $\text{dH}_2\text{O}$ ), as reported in Kobayashi et al. (2013), was selected to test these conditions due to nutrient concentrations in the digestate as compared to BBM. In the initial lab trials, 150 mL samples of anaerobically digested beef manure were utilized as algae growth media to cultivate algae under conditions of constant illumination, temperature, and agitation on an incubator-shaking unit. The initial trials were designed to compare algae growth characteristics in treatments containing 10% to 20% concentrations of anaerobically digested beef manure along with treatments utilizing autoclaved effluent or non-autoclaved effluent. No growth was observed for any samples other than autoclaved digestate or control samples (BBM).

Ghafoori and Flynn (2007) concluded that feedlots with fewer than 250,000 head of beef cattle could produce energy more economically with a centralized anaerobic digestion facility utilizing manure from multiple feedlots, while feedlots with 250,000 head or more could produce energy economically on site with anaerobic digestion. Feedlots of this size are rare, making the concept of a centralized facility more feasible in areas such as the Texas panhandle where a concentrated feedlot industry of this size exists; however, the capital cost of installation and maintenance makes neither of these methods affordable. Due to the cost prohibitive nature of anaerobic digestion and autoclaving, finding a nutrient rich waste stream that does not require a refinement process could be cost beneficial.

Runoff holding pond effluent from a feedlot is currently not viewed as a financial benefit to the feedlot. Effluent must be pumped from the basins at the feedlots to nearby fields and the irrigated effluent contains relatively low concentrations of crop nutrients,

as previously stated (Fulhage et al., 2001). In such low concentrations, the nutrients in effluent are viewed as a somewhat negligible input to crops. If the dilute nutrients in the effluent could be utilized to produce a value-added product, their value could be realized to a greater extent. Cultivation of algae is a potential process by which nutrients in runoff holding pond effluent could be economically and environmentally beneficial to the cattle operation. Therefore, the objectives of this project were to:

- (i) Quantify growth characteristics of *Chlorella sorokiniana* using varying concentrations of beef feedlot runoff holding pond effluent; and
- (ii) Determine the suitability of utilizing light absorbance quantification as a reliable predictor of algae cell density in feedstock containing beef feedlot runoff holding pond effluent.

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## CHAPTER 2: MANUSCRIPT DRAFT

### **Cultivation of *Chlorella sorokiniana* Using**

### **Beef Feedlot Runoff Holding Pond Effluent**

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### **Abstract**

Co-location of algalculture near a waste stream of nutrients is currently the only cost effective means to produce large quantities of algae. The beef industry provides an opportunity for manure nutrients to be utilized to cultivate algae. Beef feedlot runoff holding pond effluent was identified as a possible source of nutrients for algalculture co-located with a beef feedlot. A laboratory study was conducted to characterize growth of *Chlorella sorokiniana* (UTEX 1230) in vessels containing varying concentrations of holding pond effluent, with or without added nitrogen or phosphorus, and with or without autoclaving as a pretreatment. Eight treatments were randomly applied in triplicate during each of three trials. *Chlorella sorokiniana* growth in 60% pond effluent (60%PE) that had been autoclaved was statistically similar to algae growth in Bold's Basal Media (BBM), which is designed to encourage algae growth. No other treatments were autoclaved; these results suggest that the impediment to algae growth in the non-autoclaved treatments was due to competition for nutrients or predation by other microorganisms that were observed during cell density quantification. Algae cell concentration was determined my manual

enumeration using a hemocytometer and by spectrophotometry. A model built to predict cell density from light absorbance measurement illustrated that light absorbance (760 nm) may be feasible for predicting cell density at concentrations greater than 200,000 cells mL<sup>-1</sup> ( $r^2=0.75$ ).

*Keywords: algae, beef manure, cultivation, feedlot, runoff holding pond effluent*

## 2.1 Introduction

Algaculture, the large-scale production and cultivation of algae for commercial and industrial uses, has existed for more than 60 years (Borowitzka, 2013; Tamiya, 1957). Algal biomass has been used as a fertilizer (Wilkie and Mulbry, 2002; Oswald, 1988), a food product for humans (Kiple and Ornelas, 2000), and a biofuel to replace petroleum products (Bibi et al., 2017; Park et al., 2011; Pittman et al., 2011; Lin and Lin, 2011). The nutrient and energy requirements for algaculture are substantial and often cost prohibitive (Benemann, 2008). Co-location of algaculture near waste streams of nutrients, such as wastewater treatment facilities, is currently the only economically feasible method for producing algae (Pittman et al., 2011; Singh et al., 2010). Nutrients in livestock manure can be used to grow algae; success has been demonstrated using anaerobically digested poultry manure (Singh et al., 2011), fermented swine manure with chemical additives (Hu et al., 2012), and anaerobically digested beef manure (Kobayashi et al., 2013).

Nebraska has 6.45 million head of cattle, accounting for seven percent of the United States cattle inventory, with 38% of those maintained in feedlots (USDA-NASS, 2016; USDA-NRCS, 2008). Manure-laden stormwater runoff from beef feedlots is commonly collected in nearby holding ponds and stored until land application of the holding pond effluent is feasible. Because holding pond effluent typically contains dilute concentrations of nutrients (Fulhage et al., 2001), the effluent is not highly valued as a crop nutrient input. Therefore, utilizing the nutrients in the runoff holding pond effluent

to cultivate algae would not adversely affect the cattle producer or adjacent cropland and could potentially create an economic gain for the producer.

*Chlorella sorokiniana* is a single cellular freshwater algae species whose ability to grow in biological waste has been demonstrated. Kobayashi et al. (2013) identified *Chlorella sorokiniana* as being an ideal algae strain to grow using a 10% solution of digestate from anaerobically digested beef manure. The high protein content, high carbohydrate content, and low lipid content of *C. sorokiniana* makes it a potential candidate as feedstock for beef cattle (Kobayashi et al., 2013; Hu et al., 2012). Given the potential for *C. sorokiniana* to be cultivated from beef feedlot holding pond effluent, and its potential value as a cattle feed additive, the objectives of this research were to 1) quantify growth characteristics of *Chlorella sorokiniana* using varying concentrations of beef feedlot runoff holding pond effluent with and without nutrient supplementation and pre-treatment by autoclaving; and 2) determine the suitability of utilizing light absorbance quantification as a reliable predictor of algae cell density in feedstock containing beef feedlot runoff holding pond effluent.

## **2.2 Materials and Methods**

### **2.2.1 Feedlot Holding Pond Effluent**

Effluent was obtained from the runoff holding ponds at ten commercial beef feedlot operations in primarily eastern and central Nebraska. To obtain samples, kits were shipped to cooperating beef producers with instructions to fill three 1-L HDPE bottles and one 500-mL HDPE bottle with effluent dipped from the top 15 to 30 cm of liquid in their feedlot runoff holding pond and return samples to the University of Nebraska –

Lincoln via pre-paid overnight shipping immediately following collection. Samples were received from cooperating producers between May and June of 2016. Upon receipt of samples from farms, one 1-L bottle from each site was immediately placed into refrigerated storage at 4°C while the other two 1-L bottles were placed into storage at -20°C. The 500-mL sample from each site was submitted to a commercial laboratory for nutrient analysis. A composite was created using 600 mL of effluent from each site, sub-sampled, and submitted to a commercial laboratory for nutrient analysis. Data provided by each cooperating producer included: feedlot capacity (head), date and time of sample collection, holding pond capacity (liters), and volume of pond at time of sampling (liters).

### **2.2.2 Algae Strain and Growth Conditions**

*C. sorokiniana* UTEX 1230 was obtained from the University of Texas at Austin and maintained on the agar slant under cool white fluorescent light at approximately 20°C until cultivation. *C. sorokiniana* was cultivated in the lab by transferring the algae using a sterile spatula from the agar slant into 50 mL of Bold's Basal Media (BBM) contained in a sterile 100 mL beaker. The beaker was continuously agitated on a Benchmark Incu-Shaker 10L (Benchmark Scientific, Sayreville, New Jersey) at 30°C and 140 RPM. Once the cell density in the algae culture reached approximately 10 to 15 million cells-mL<sup>-1</sup> as determined by manual enumeration using a hemocytometer (Bright Line Counting Chamber, Hausser Scientific, Horsham, PA) and microscope (B2-Series, Fisher Scientific, Waltham, MA), the culture was transferred to a 1 L sterile beaker and diluted 1:10 with BBM. This process was repeated until a 1 L culture with approximately 10 to 15 million cells-mL<sup>-1</sup> was achieved.

### 2.2.3 Application of Treatments

Eight treatments were prepared for cultivation of algae during each of three independent trials: 150 mL runoff holding pond effluent (PE) (100%PE); 90 mL PE + 60 mL distilled water (dH<sub>2</sub>O) (60%PE); 90 mL PE + 60 mL dH<sub>2</sub>O + 0.0375 g NaNO<sub>3</sub> (60%PEN); 90 mL PE + 60 mL dH<sub>2</sub>O + 0.0113 g K<sub>2</sub>HPO<sub>4</sub> + 0.0263 g KH<sub>2</sub>PO<sub>4</sub> (60%PEP); 90 mL PE + 60 mL dH<sub>2</sub>O + 0.0375 g NaNO<sub>3</sub> + 0.0113 g K<sub>2</sub>HPO<sub>4</sub> + 0.0263 g KH<sub>2</sub>PO<sub>4</sub> (60%PENP); 90 mL autoclaved PE + 60 mL dH<sub>2</sub>O (60%PEAC); 30 mL PE + 120 mL dH<sub>2</sub>O (20%PE); and 150 mL Bold's Basal Media (BBM) (Bischoff and Bold, 1963) as a control. Distilled water (dH<sub>2</sub>O) used for dilutions was obtained from a Milli-Q Direct-8 water purifier (EMD Millipore Corporation, Billerica, MA). The additions of NaNO<sub>3</sub>, K<sub>2</sub>HPO<sub>4</sub>, and KH<sub>2</sub>PO<sub>4</sub> were performed to yield nitrogen and phosphorus concentrations in the holding pond effluent treatments equivalent to the concentration of these nutrients in BBM.

For each trial, twenty-four 200-mL glass beakers were sterilized in a Tuttnauer Brinkmann 3850E autoclave (Tuttnauer Co. Ltd., Beit Shemesh, Israel) for 3 minutes at 134°C. Sterilized graduated cylinders and tools were used to distribute treatments among the beakers with each treatment replicated in triplicate during each trial. Algae culture was introduced to treatment beakers by pipetting 5 mL of stock algae at 12 million cells-mL<sup>-1</sup>, 10 million cells-mL<sup>-1</sup>, and 14.4 million cells-mL<sup>-1</sup> for trials 1, 2, and 3, respectively, using sterile methods.

Beakers were randomly arranged in a Benchmark Incu-Shaker 10L (Benchmark Scientific, Sayreville, NJ) maintained at a target temperature of 30°C and agitated at 140 rpm for 24 days. Continuous light was supplied by two 360-lumen LED rechargeable



handheld work lights (Smart Electrician, Menards, Inc., Eau Claire, WI). To account for daily evaporation, purified water was added to beakers daily to return the liquid levels to a fill line drawn on the outside of each beaker on day one.

#### **2.2.4 Sample Collection and Preparation**

Daily sample collection from each beaker was initiated ten minutes following water addition to account for evaporation. One mL of liquid was collected from each beaker at a depth of 3 cm via a Thermo Scientific Finn timer F1 100-1000  $\mu$ L Pipette (Thermo Fisher Scientific, Waltham, MA) and transferred to a sterile 30 mL beaker. Nine mL of dH<sub>2</sub>O was added to the sample with a Fisherbrand Elite 1-10 mL Pipette (Thermo Fisher Scientific, Waltham, MA) and the sample was manually agitated for 5 s.

#### **2.2.5 Algae Cell Quantification**

Manual cell counting and measurement of light absorbance by spectrophotometer were performed on all samples. For manual cell counting, ten  $\mu$ L of the diluted sample was pipetted with a Finn timer F1 2-20  $\mu$ L pipette (Thermo Fisher Scientific, Waltham, MA) onto a hemocytometer (Bright Line Counting Chamber, Hausser Scientific, Horsham, PA). The hemocytometer was placed on a Fisher Scientific Microscope B2-Series Model S71012A (Fisher Scientific, Waltham, MA) equipped with a 5-megapixel Motic camera, allowing for projection of microscope images onto a computer monitor. Images were captured and recorded on a microSD card. Algae cells were enumerated within five areas on the hemocytometer (Figure 1) using the computer images (Figure 2).

Three mL of the remaining diluted sample were pipetted with a Finn timer F1 100-1000  $\mu$ L pipette (Thermo Fisher Scientific, Waltham, MA) into a cuvette and placed

into a Genesys 20 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) to measure light absorbance of samples. The spectrophotometer was calibrated at a wavelength of 760 nm based upon measured light absorbance of a 3-mL cuvette of purified water. Light absorbance at 760 nm was recorded for each sample.

### **2.2.6 Preliminary Trials**

Initial laboratory studies were conducted using digestate from the anaerobic digestion of beef manure following the methods and materials presented in this paper. This prior research attempted to replicate results observed by Kobayashi et al. (2013) during cultivation of *C. sorokiniana* in hanging bags using anaerobic digested beef manure. However, this study differed from Kobayashi et al. (2013) by simulating open pond algae production with beakers under constant agitation rather than using hanging bags. The method yielded dissimilar results to Kobayashi et al. (2013) in that the raw beef manure digestate did not promote algae growth beyond approximately four days. Autoclaved anaerobically digested beef manure did support algae growth for the duration of the trials, achieving a maximum cell density of approximately 11 million cells-mL<sup>-1</sup>. The control used in these preliminary trials (Bold's Basal Media) achieved a maximum cell density of nearly 30 million cells-mL<sup>-1</sup>. These results suggested that algae were either outcompeted for nutrients by other microorganisms in the digestate or were serving as a food source for other microorganisms. Based on these results, a new direction was pursued to investigate cultivation of algae using runoff holding pond effluent, which is readily available on most beef feedlot operations and does not require additional costly infrastructure like that needed for anaerobic digestion.

### 2.2.7 Statistical Analysis

Mean manual cell counts and light absorbance values by treatment and day across all replications ( $n=3$ ) and trials ( $n=3$ ) were plotted and calculation of the area under the curve (AUC) for each was performed using the AUC function (Ballings and Van den Poel, 2015) in R (R Core Team, 2013). The GLIMMIX procedure in SAS (SAS Institute Inc., Cary, NC) was used to identify differences ( $p < 0.05$ ) in calculated AUC values among treatments. The GLIMMIX procedure in SAS (SAS Institute Inc., Cary, NC) was also used to identify differences ( $p < 0.05$ ) in day 24 cell density and light absorbance among all treatments. A regression model was developed using the REG procedure in SAS (SAS Institute Inc., Cary, NC) to predict cell density from light absorbance value.

## 2.3 Results and Discussions

### 2.3.1 Characterization of Feedlot Holding Pond Effluent

Effluent collected from runoff holding ponds at ten Nebraska feedlots in Spring 2016 were analyzed for nutrients and other chemical characteristics as reported in Table 1. As anticipated, variability in nutrient concentrations among the samples was greatest for macronutrients, including organic nitrogen (Org N), ammonium ( $\text{NH}_4$ ), nitrate ( $\text{NO}_3$ ), total Kjeldahl nitrogen (TKN), phosphate ( $\text{P}_2\text{O}_5$ ) and potash ( $\text{K}_2\text{O}$ ), with coefficient of variation among micronutrients, pH and total solids being less pronounced. Dickey and Vanderholm (1977) reported beef feedlot runoff nutrient concentrations similar to the TKN and  $\text{P}_2\text{O}_5$  observed in this study. Kreis et al. (1972) reported similar nutrient concentrations for TKN, magnesium (Mg), and potassium (K) as reported in this paper. The nutrients reported in the composited sample are representative of nutrient

concentrations seen in other literature, though deviation in values exist. Some papers did not report nutrients that were measured in this research. Management practices, precipitation events, and stocking density are important factors impacting runoff holding pond effluent characteristics (Dickey and Vanderholm, 1977). Given the spatial and temporal variability among sampling events, variability in feedlot sizes and management systems, and individual collection strategies employed by the individuals submitting samples, the high standard deviations (SD) for many of the reported characteristics are unsurprising. Characteristics of the composite sample created from the submitted samples (n=10), which was utilized for algae cultivation, are shown in Table 2.

### 2.3.2 Algae Biomass Production

*C. sorokiniana* UTEX 1230 was grown over three 24-d trials under eight treatments replicated in triplicate during each trial: 150 mL runoff holding pond effluent (PE) (100%PE); 90 mL PE + 60 mL distilled water (dH<sub>2</sub>O) (60%PE); 90 mL PE + 60 mL dH<sub>2</sub>O + 0.0375 g NaNO<sub>3</sub> (60%PEN); 90 mL PE + 60 mL dH<sub>2</sub>O + 0.0113 g K<sub>2</sub>HPO<sub>4</sub> + 0.0263 g KH<sub>2</sub>PO<sub>4</sub> (60%PEP); 90 mL PE + 60 mL dH<sub>2</sub>O + 0.0375 g NaNO<sub>3</sub> + 0.0113 g K<sub>2</sub>HPO<sub>4</sub> + 0.0263 g KH<sub>2</sub>PO<sub>4</sub> (60%PENP); 90 mL autoclaved PE + 60 mL dH<sub>2</sub>O (60%PEAC); 30 mL PE + 120 mL dH<sub>2</sub>O (20%PE); and 150 mL Bold's Basal Media (BBM) (Bischoff and Bold, 1963) as a control.

Initially, all of the treatments except the BBM were opaque and brown in color (Figure 3). As the trials progressed, solids settled in the treatments containing PE and these treatments became clearer and lighter brown in color (Figure 4 and 5). The BBM treatment was clear at the onset of the trials and dark green at the conclusion of the trials.

Manual quantification of cells was performed daily throughout all three 24-d trials using a hemocytometer and microscope. Cell quantity values recorded for five areas on the hemocytometer (Figure 1) were averaged. Each cell counted on the hemocytometer represented  $100,000 \text{ cells-mL}^{-1}$  in the sample when accounting for dilution.

Mean ( $n=9$ ) cell density by treatment across all trials is illustrated in Figure 6. During the initial days of each trial, all treatments showed similar concentrations of algae cells. After day 5, cell densities for 60%PEAC and BBM begin to steadily increase while the remaining treatments experienced decreases in cell densities (Figure 6). Positive growth was maintained in the 60%PEAC and BBM treatments throughout the trials ( $n=9$ ), while all other treatments showed no continued algae growth after day 5.

Values for AUC from hemocytometer counting data were 1537.03, 1259.97, 641.63, 583.40, 456.61, 448.17, 400.83, and 380.00 [ $100,000 \text{ cells-day-mL}^{-1}$ ] for BBM, 60%PEAC, 60%PENP, 60%PEN, 60%PEP, 60%PE, 20%PE, and 100%PE, respectively. Statistical analysis of cell density by hemocytometer counting among treatments, shown in Table 3, indicates that, when using the AUC method described above, the BBM and the 60%PEAC treatments were statistically different from the other treatments and were statistically similar to one another. This means the algal growth observed in both the BBM and 60%PEAC was significantly greater than the other treatments. Cell densities on day 24 were 125.60, 44.84, 0.84, 0.53, 0.49, 0.44, 0.38, and 0.29 [ $100,000 \text{ cells-mL}^{-1}$ ] for BBM, 60%PEAC, 20%PE, 60%PEP, 60%PENP, 60%PEN, 60%PE, 100%PE respectively. Analysis of day 24 cell density by hemocytometer counting among treatments, shown in Table 4, indicates that both 60%PEAC and BBM are statistically different from all other treatments, but are also statistically different from one another.

This reveals that both treatments showed significantly greater algae growth throughout the trials, but the observed cell densities on the final day of cultivation were not sufficient to conclude that BBM and 60%PEAC treatments yielded similar results, with 125.60 and 44.84 [100,000 cells-mL<sup>-1</sup>] for BBM and 60%PEAC, respectively.

All treatments, including the control (BBM), experienced a “crash,” or complete culture death, in one or more replicates throughout the three-trial study. Three of the nine BBM treatment replicates (2, 0, and 1, in trials 1, 2 and 3, respectively) experienced algae culture death prior to day 24, typically occurring rapidly following a daily sample collection, which is hypothesized as being due to inadvertent introduction of a contaminant or other human error. Algae culture death also occurred in six of nine 60%PEAC cultures (2, 0, and 1, in trials 1, 2 and 3, respectively). To more accurately represent and compare algae growth trends among the BBM and 60%PEAC, replicates from these treatments that experienced culture death prior to day 24 were excluded from the data sets, yielding the mean cell density plots for BBM (n=6) and 60%PEAC (n=6) in Figure 7. Similar growth trends are evident among each of the treatments when comparing Figures 6 and 7; however, mean cell density is greater for each treatment in Figure 7. Mean cell density for the BBM treatment on day 24 increased from 13 million cells-mL<sup>-1</sup> to 19 million cells-mL<sup>-1</sup> with removal of the replicates experiencing cell culture death. Likewise, the mean cell density for the 60%PEAC treatment on day 24 increased from 5 million cells-mL<sup>-1</sup> to 7 million cells-mL<sup>-1</sup> with removal of the replicates experiencing cell culture death.

Mean (n=9) light absorbance by treatment across all trials is illustrated in Figure 8. During the initial days of each trial, light absorbance was artificially elevated for all

treatments containing PE due to suspended solids. The light absorbance over time for the 100%PE treatment, which yielded the highest value for light absorbance on day 1, reflects the effect of suspended solids on light absorbance and illustrates the settling of solids throughout the trial period as a steady decline in light absorbance is observed. The BBM treatment initially yielded the lowest light absorbance among treatments, indicating the absence of suspended solids contributed by PE. These results support the assertion that light absorbance will be of limited value as an indicator of algae cell density in solutions containing suspended solids, such as the runoff holding pond effluent used in this study.

Mean AUC values by treatment for plotted light absorbance trends (Table 5) indicate that 100%PE, 60%PEAC, and BBM did not differ ( $p<0.05$ ) from one another. The mean AUC for 20%PE and 60%PENP were also not different ( $p<0.05$ ). However, a comparison among mean light absorbance on day 24 (Table 6) indicates that BBM is statistically different from all other treatments and 60%PEAC is statistically different from all treatments except 100%PE ( $p<0.05$ ). These contradicting results suggest, in agreement with results discussed in the previous paragraph, that total suspended solids (TSS) in PE skew light absorbance values such that TSS and algae cannot be differentiated. As such, it appears that light absorbance alone may not provide an accurate indication of algal cell density for solutions containing suspended solids.

### **2.3.3 Comparison of Cell Counting and Light Absorbance**

Counting cells manually to determine algal culture density is time-consuming and tedious. Therefore, a comparison was made between manual cell counts and light absorbance measurements for samples from this study to determine if a trend could be

established. Prediction of cell density from light absorbance measured via spectrophotometry has been demonstrated for other algae substrates by Isleten-Hosoglu et al. (2012), Fu et al. (2012), and Converti et al. (2009).

Light absorbance was plotted against manually enumerated cell density for all samples (n=1719) (Figure 9). Using SAS (SAS Institute Inc., Cary, NC) a Pearson correlation coefficient of 0.67 was calculated to identify the presence of a trend, either positive or negative. This value is on a scale from -1 to 1, where 0 indicates no correlation, 1 indicates a perfect positive correlation, and -1 indicates a perfect negative correlation. The correlation of 0.67 shows a moderate-strength positive trend. As mentioned previously, error is introduced to spectrophotometer readings when suspended sediment is present in the sample, artificially increasing cell count prediction. Additionally, sample dilution at low initial cell density results in reduced spectrophotometer measurement resolution. Therefore, data points representing manual cell density values less than 200,000 cells-mL<sup>-1</sup> were removed from the data set and a Pearson correlation coefficient was again calculated. For the data points based upon manual cell density values greater than 200,000 cells-mL<sup>-1</sup> (Figure 10), a Pearson correlation coefficient of 0.86 was calculated, indicating a much stronger correlation.

A regression equation was generated to predict cell density based upon OD<sub>760</sub> using the data points in Figure 10.

$$y = 3964.7x - 6.283 \quad \text{Eqn. 1}$$

An R-Squared (r<sup>2</sup>) value of 0.75 for this regression equation suggests that approximately 75% of the variability in cell density value can be directly explained by its relationship to



light absorbance as determined by spectrophotometry. Fu et al. (2012), Converti et al. (2009), and Isleten-Hosoglu et al. (2012) each created a model similar to Equation 1 with  $r^2$  values of 0.99, 0.99, and 0.95, respectively, based upon algae growth reported as a dry weight rather than individual cell enumeration.

#### **2.3.4 Competition for Nutrients**

One treatment, 60%PEAC, was autoclaved to eliminate microorganisms present in the effluent that presented potential competition with algae for nutrients. Nutrient analyses of the pond effluent before and after autoclaving were obtained to determine if the autoclaving process affected nutrient availability. Relatively little change in effluent characteristics resulted from autoclaving (Table 7). Nitrate-N and pH both increased with autoclaving compared to the non- autoclaved sample while ammonium-N concentration decreased. Nitrification of ammonium-N to nitrate-N represents an increase in availability of the nitrogen, which should be beneficial to algae growth. Anderson and Magdoff (2005) found that autoclaving soil can cause an increase in algal-available phosphorus, but not significantly higher than non-autoclaved soils based on observed algal growth in their samples. The greater algae growth observed for the 60%PEAC treatment compared to other treatments containing non-autoclaved effluent, therefore, does not appear to be significantly influenced by changes in nutrient resulting from autoclaving.

The most reasonable explanation for significantly greater algal growth observed in the autoclaved sample versus the non-autoclaved samples is competition for nutrients by other organisms present in the non-autoclaved effluent. Numerous organisms were observed in the non-autoclaved samples during manual cell enumeration under a

microscope. Images displayed in Figure 11 represent organisms that were photographed during manual cell enumeration on the hemocytometer and were observed moving and interacting under the microscope. While quantification and analysis of these organisms were not pursued, identification of two organisms – *Testudinella* sp., a rotifer, (Figure 11a) and an amoeba (Figure 11b) – revealed that these microorganisms are algae consumers.

## 2.4 Conclusions

The only treatment that showed a statistically similar ( $p < 0.05$ ) concentration of *C. sorokiniana* to the control (BBM) treatment was 60%PEAC, while algae concentrations in remaining treatments were significantly less ( $p < 0.05$ ). The correlation between cell density and light absorbance at 760 nm yielded an R-squared ( $r^2$ ) value of 0.75, demonstrating that light absorbance may provide an acceptable prediction of algal cell density for *Chlorella sorokiniana* (UTEX 1230) grown in beef feedlot holding pond effluent when cell density is greater than 200,000 cells-mL<sup>-1</sup>. Suspended solids in the effluent, however, limit the suitability of the regression model to accurately predict cell density for this experimental design at a cell density below 200,000 cells-mL<sup>-1</sup>.

The results of this study suggest that raw (non-autoclaved) beef feedlot runoff holding pond effluent is not capable of sustaining growth of *C. sorokiniana*. Competition for nutrients by microorganisms found in the effluent and consumption of algae by these microorganisms are likely responsible for the insignificant algae growth documented in all treatments containing non-autoclaved beef feedlot runoff holding pond effluent.

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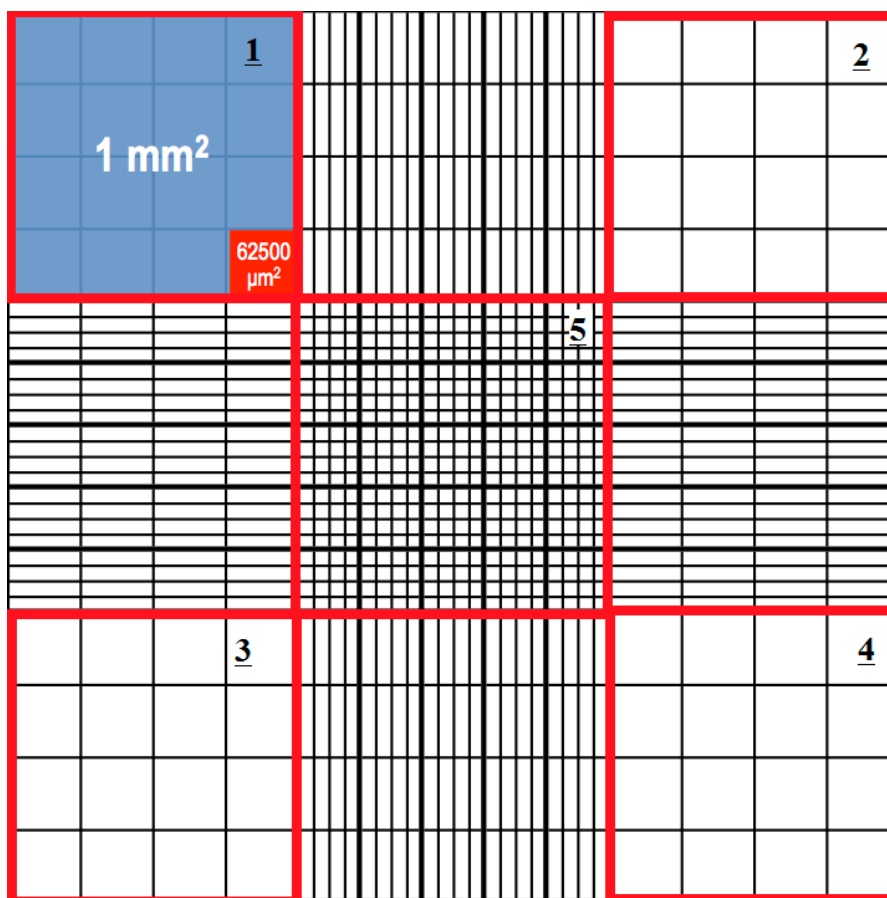
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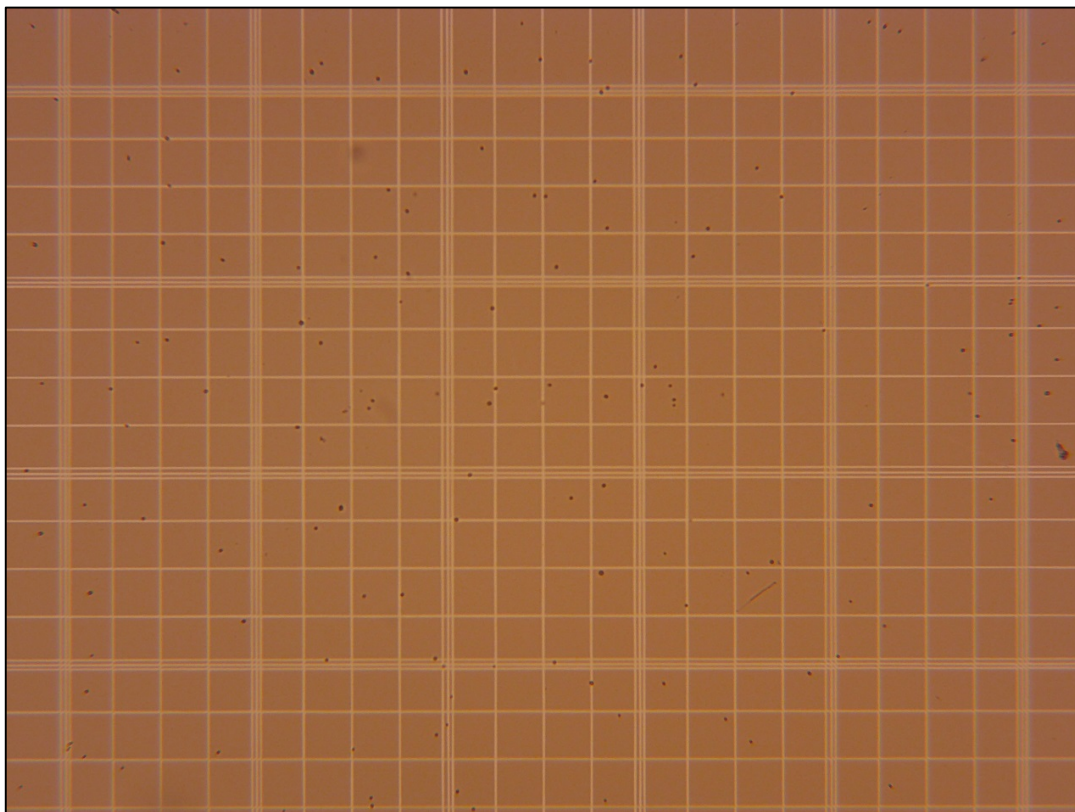
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## 2.7 Appendix

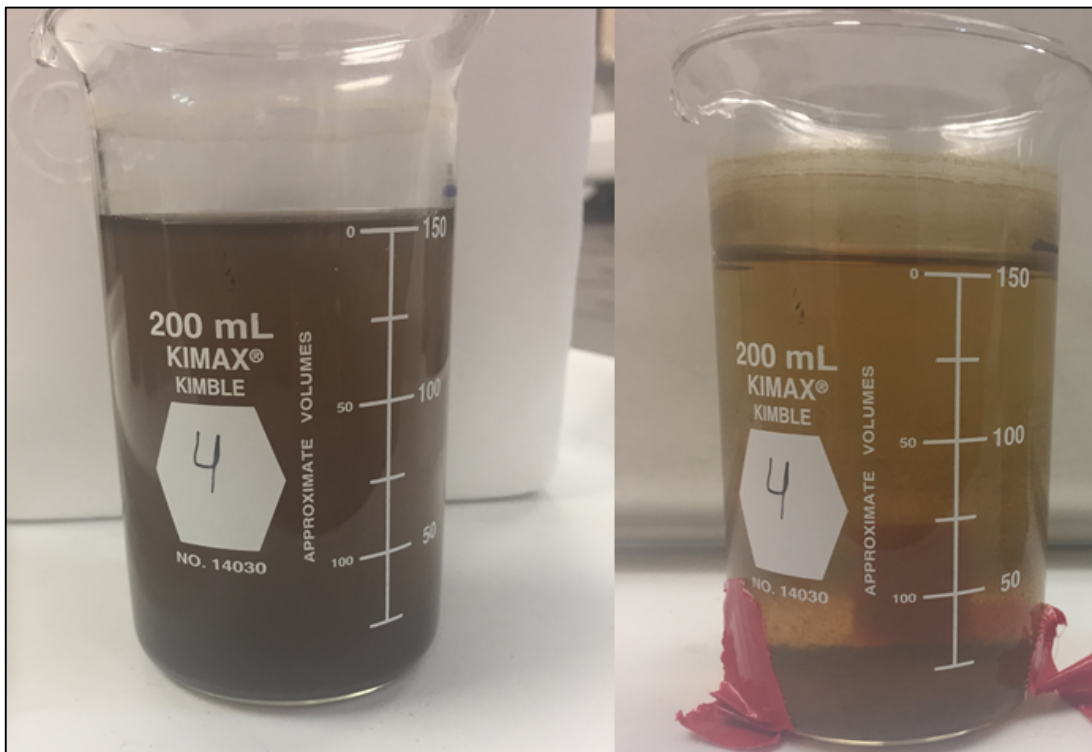


**Figure 1. Hemocytometer layout illustrating five sections, each 1 mm x 1 mm, used for enumeration of cells during manual cell counting.**





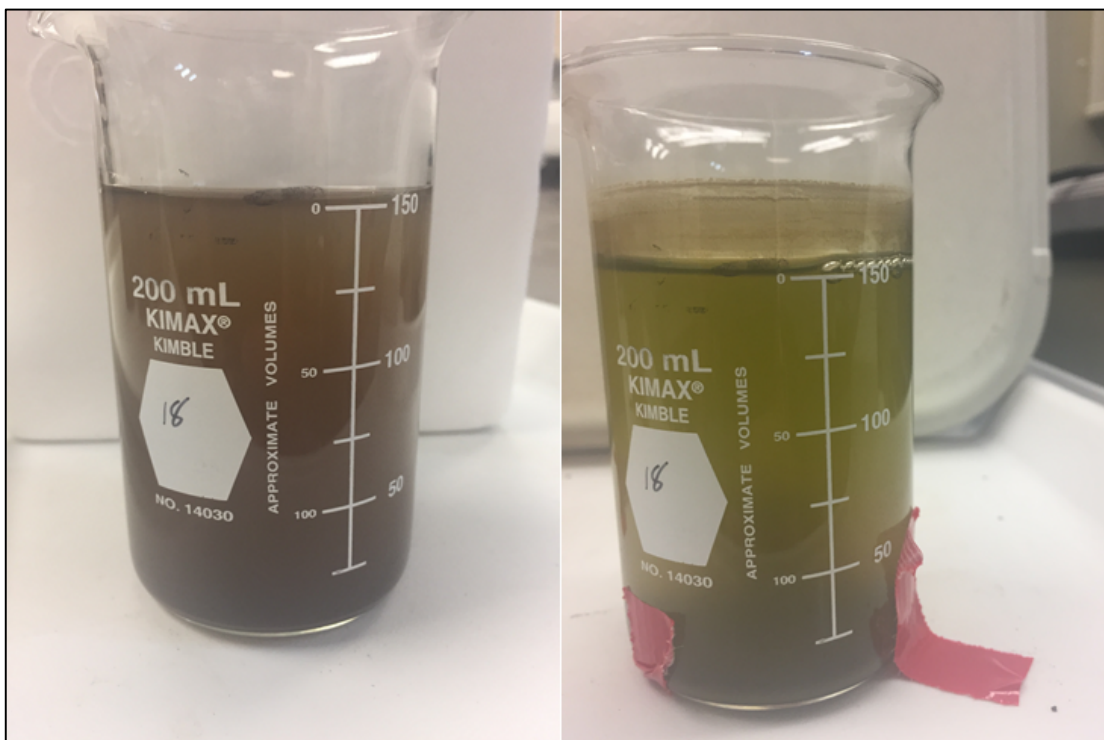
**Figure 2. Hemocytometer image used to count cells.**



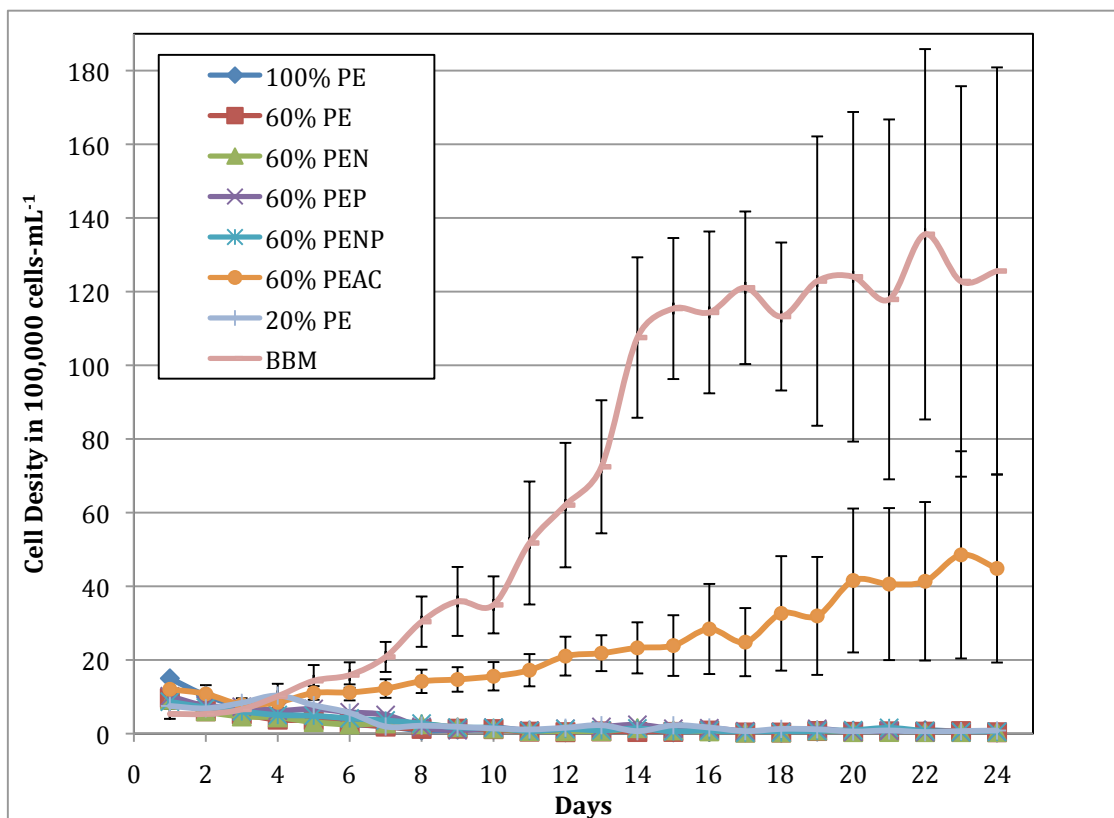
**Figure 3. One replicate of the 60%PE treatment from trial three illustrating the appearance of the culture on day 0 (L) and day 24 (R).**



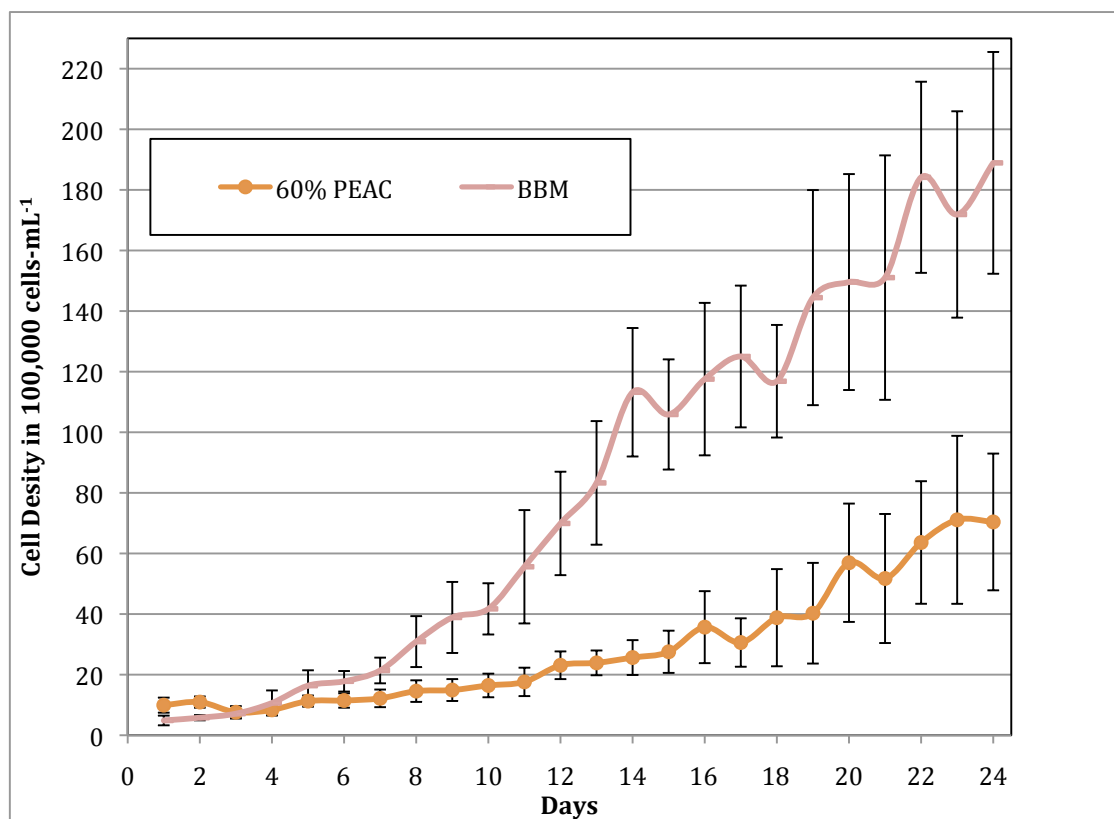
**Figure 4. One replicate of the BBM treatment from trial two illustrating the appearance of the culture on day 0 (L) and day 24 (R).**



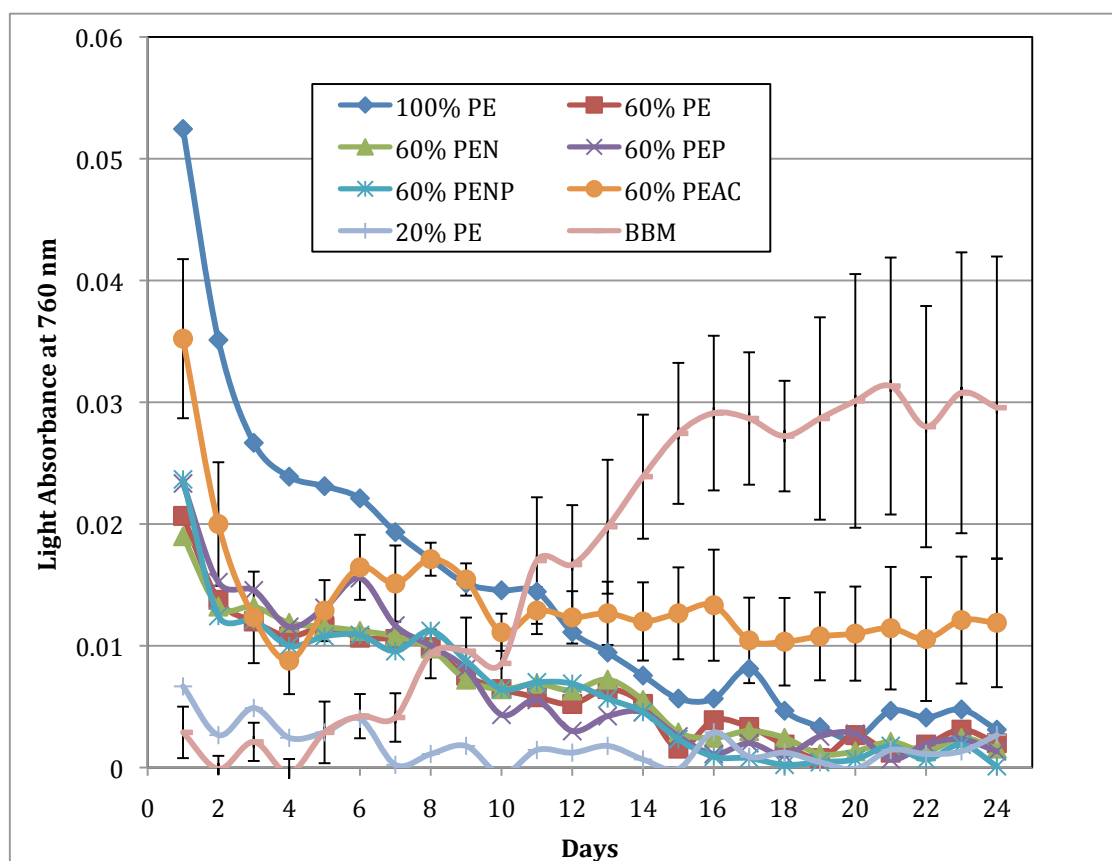
**Figure 5. One replicate of the 60%PEAC treatment from trial three illustrating the appearance of the culture on day 0 (L) and day 24 (R).**



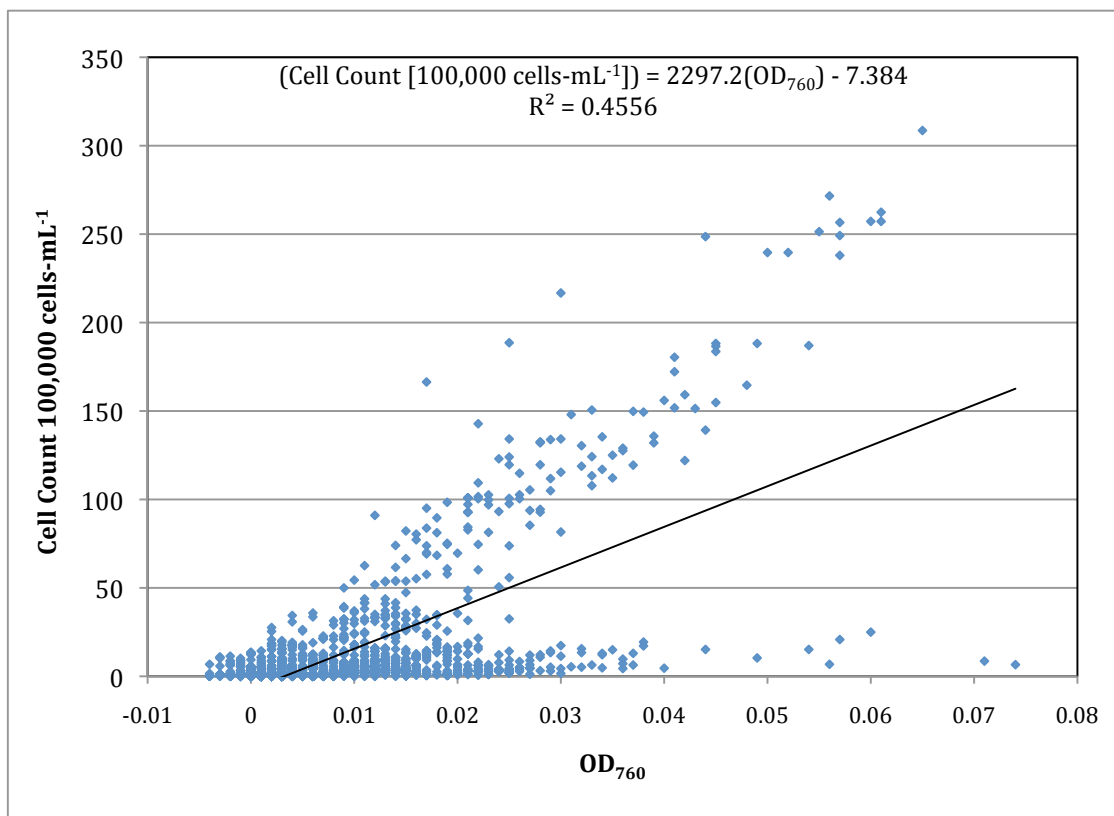
**Figure 6. Mean (n=9) algae cell concentration (100,000 cells·mL<sup>-1</sup>) over time, by treatment, across all trials as determined by manual cell counting.**



**Figure 7. Mean (n=6) cell density over time for 60%PEAC and BBM treatments, as determined by manual cell counting.**

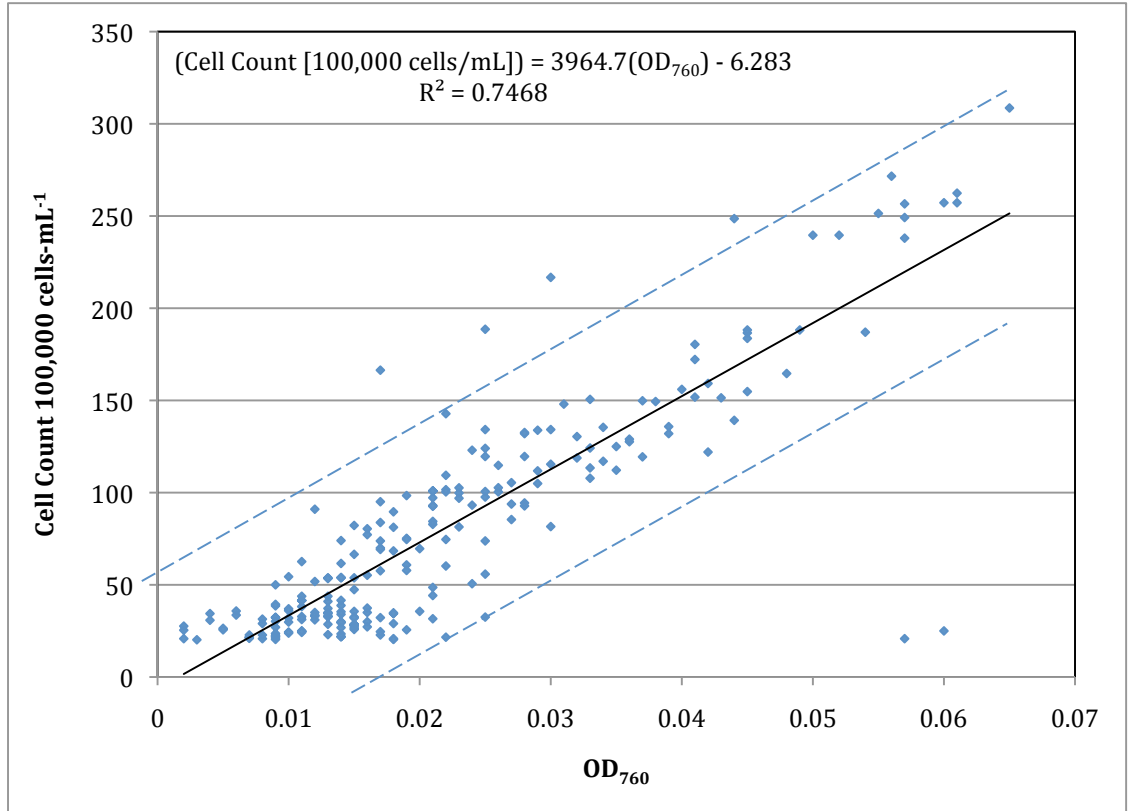


**Figure 8. Mean (n=9) light absorbance at 760 nm of samples over time, by treatment, across all trials. Error bars indicate standard deviation.**

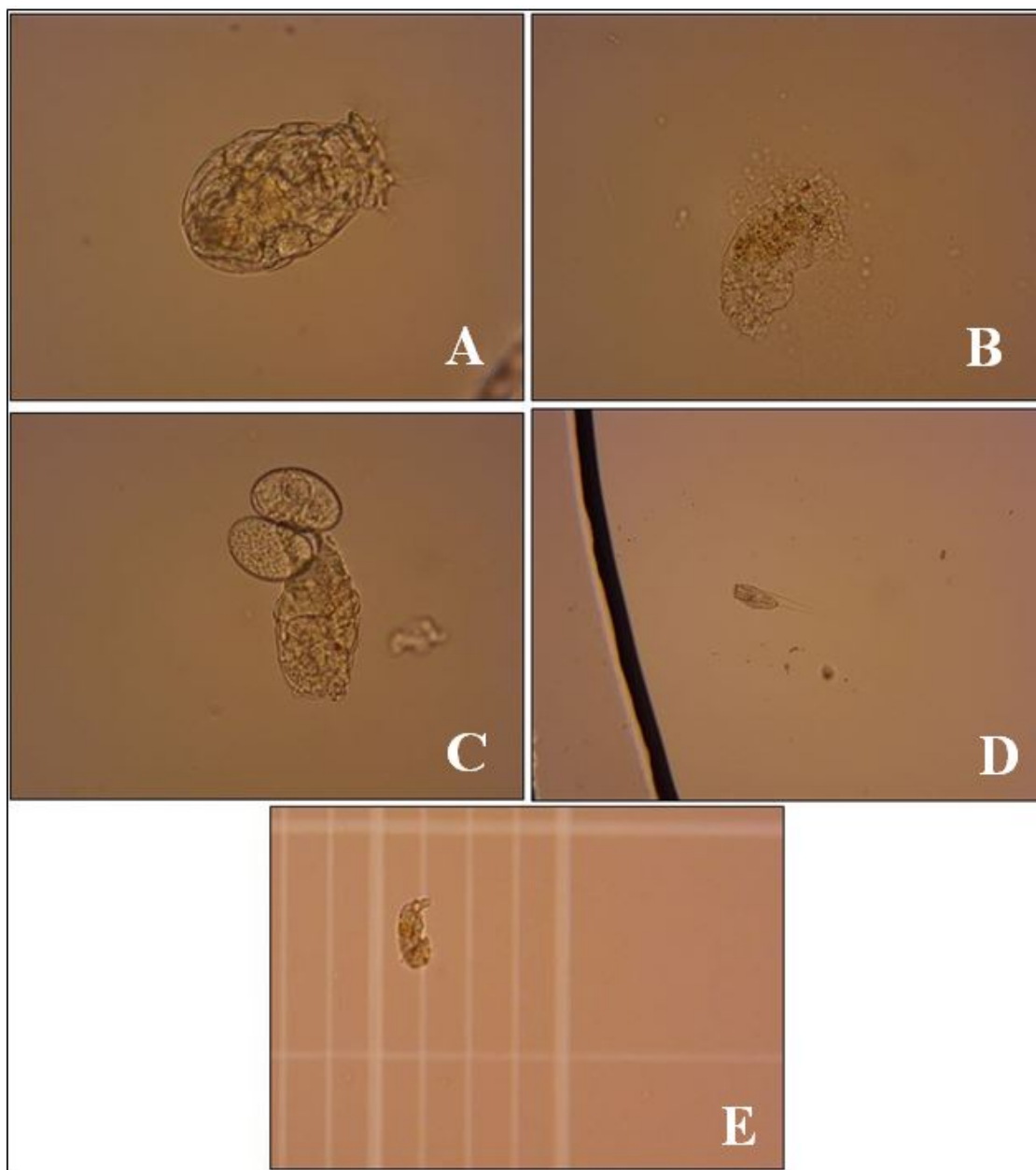


**Figure 9. Relationship between measured light absorbance (760 nm) and manually enumerated cell density for all data points (n=1719). The absorbance equation is represented by the solid line: {(Cell Count [100,000 cells/mL]) = 2297.2( $OD_{760}$ ) - 7.384} ( $r^2=0.4556$ ).**





**Figure 10. Relationship between absorbance (760 nm) and cell density for *Chlorella sorokiniana* solutions for cell density greater than 2,000,000 cells-mL<sup>-1</sup>. Data points (blue dots) represent values obtained from manual cell quantification (n=223). The absorbance equation is represented by the solid line: {(Cell Count [100,000 cells/mL])=3964.7( $OD_{760}$ )-6.283} ( $r^2=0.7468$ ). Dotted lines show the prediction limits for the model.**



**Figure 11. Microorganism images captured during manual cell enumeration for treatments containing unautoclaved pond effluent; (a) rotifer *Testudinella*; (b) amoeba; and (c through e) unidentified microorganisms**

Table 1. Runoff holding pond sample characteristics

Feedlot Number	Location in NE	Organic N	Ammonium NH <sub>4</sub>	Nitrate NO <sub>3</sub>	Total N TKN	Phosphorus P <sub>2</sub> O <sub>5</sub>	Potassium K <sub>2</sub> O	ppm										Soluble Salts	pH	Total Solids %
								Sulfur	Calcium	Magnesium	Sodium	Zinc	Iron	Manganese	Copper	Boron				
1	Central	60.10	107.80	0.90	168.90	177.20	880.50	43.90	109.80	45.60	200.90	1.10	20.40	0.70	0.40	0.40	4.08	7.30	0.42	
2	Central	47.90	67.90	0.20	116.10	125.20	633.70	31.60	121.40	60.20	186.10	0.30	7.60	0.40	0.09	0.20	3.28	7.40	0.28	
3	Western	59.20	9.70	1.10	70.00	16.60	66.10	70.50	125.10	31.60	172.10	0.20	9.70	0.20	0.10	0.00	1.11	8.10	0.12	
4	Western	34.70	6.50	0.80	42.00	12.40	60.00	64.80	86.00	28.40	149.00	0.10	5.50	0.10	0.09	0.00	1.07	8.10	0.08	
5	Western	74.90	64.70	0.90	140.50	78.60	313.90	28.80	227.70	76.10	179.00	0.50	11.70	0.30	0.09	0.50	2.88	7.70	0.22	
6	Western	258.70	136.70	5.90	401.20	217.50	1037.50	94.30	429.70	208.10	325.70	2.10	53.20	2.60	0.50	1.00	6.45	7.70	0.82	
7	Western	175.30	138.50	1.00	314.80	191.10	1025.00	92.80	338.70	188.10	322.40	1.50	320.00	1.80	0.40	0.90	6.43	7.80	0.68	
8	Western	230.50	477.60	1.30	709.40	160.30	3386.70	89.50	398.70	216.80	523.10	2.50	24.20	2.00	0.60	1.00	17.43	7.90	1.21	
9	Central	61.80	23.30	3.40	88.50	48.80	295.10	51.50	127.70	47.80	91.50	0.20	6.20	0.30	0.09	0.20	2.10	7.60	0.21	
10	Eastern	30.50	3.20	1.40	350.00	15.30	216.40	25.90	138.60	47.60	93.50	0.20	5.20	0.10	0.10	0.50	1.18	7.90	0.08	
Coeff. Var.		0.82	1.36	1.01	1.02	0.76	1.25	0.45	0.62	0.81	0.59	0.98	0.88	1.08	0.81	0.83	1.07	0.03	0.92	
Average		103.36	103.59	1.69	208.64	104.3	791.49	59.36	210.34	95.03	224.33	0.90	17.60	0.85	0.25	0.47	4.60	7.80	0.412	
Median		60.95	66.30	1.05	128.3	101.9	473.8	58.15	133.15	54.00	182.55	0.40	10.70	0.35	0.1	0.45	3.08	7.80	0.25	

Table 2. Nutrient characteristics of composited holding pond effluent samples (n=10)

	Organic N	Ammonium	Nitrate	Total N	Phosphorus	Potassium	Sulfur	Calcium	Magnesium	Sodium	Zinc	Iron	Manganese	Copper	Boron	Soluble Salts	pH	Dry Matter %

Composited Sample

ppm

210.7	97.9	2.5	311.1	79.9	610.7	50.5	174.6	76.0	202.5	0.7	14.4	0.9	1.3	0.4	4.2	7.8	0.4
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**Table 3. Mean area under the curve (AUC) by treatment for hemocytometer cell density values**

<b>Treatment</b>	<b>AUC</b>
BBM	1537.03 <sup>a</sup>
60%PEAC	1259.97 <sup>a</sup>
60%PENP	641.63 <sup>b</sup>
60%PEN	583.40 <sup>b</sup>
60%PEP	456.61 <sup>b</sup>
60%PE	448.17 <sup>b</sup>
20%PE	400.83 <sup>b</sup>
100%PE	380.00 <sup>b</sup>

Values in the same column followed by different superscripts are significantly different at the 0.05 probability level based on the LSD test.

**Table 4. Mean cell density on day 24 as determined by hemocytometer counting**

<b>Treatment</b>	<b>Cell Density (cells-mL<sup>-1</sup>)</b>
BBM	125.60 <sup>a</sup>
60%PEAC	44.84 <sup>b</sup>
20%PE	0.84 <sup>b</sup>
60%PEP	0.53 <sup>c</sup>
60%PENP	0.49 <sup>c</sup>
60%PEN	0.44 <sup>c</sup>
60%PE	0.38 <sup>c</sup>
100%PE	0.29 <sup>c</sup>

Values in the same column followed by different superscripts are significantly different at the 0.05 probability level based on the LSD test.

**Table 5. Mean area under the curve (AUC) by treatment for light absorbance**

<b>Treatment</b>	<b>AUC</b>
BBM	0.3508 <sup>a</sup>
60%PEAC	0.3100 <sup>a</sup>
100%PE	0.3068 <sup>a</sup>
60%PEP	0.1643 <sup>b</sup>
60%PEN	0.1507 <sup>b</sup>
60%PE	0.1382 <sup>b</sup>
60%PENP	0.1342 <sup>b,c</sup>
20%PE	0.0328 <sup>c</sup>

Values in the same column followed by different superscripts are significantly different at the 0.05 probability level based on the LSD test.

**Table 6. Mean (n=9) light absorbance by treatment on day 24**

<b>Treatment</b>	<b>Estimate</b>
BBM	0.029560 <sup>a</sup>
60%PEAC	0.011890 <sup>b</sup>
100%PE	0.003111 <sup>b</sup>
60%PE	0.002000 <sup>c</sup>
60%PEN	0.001556 <sup>c</sup>
60%PEP	0.001333 <sup>c</sup>
20%PE	0.001143 <sup>c</sup>
60%PENP	0.000111 <sup>c</sup>

Values in the same column followed by different superscripts are significantly different at the 0.05 probability level based on the LS-Means test.



Table 7. Characteristics of pooled (n=10) runoff holding pond effluent samples prior to and following autoclaving

	Organic N	Ammonium	Nitrate	Total N	Phosphorus	Potassium	Sulfur	Calcium	Magnesium	Sodium	Zinc	Iron	Manganese	Copper	Boron	Soluble Salts	pH	Dry Matter %
Autoclaved	119.8	50.1	9.4	179.3	83.8	1011.9	51.4	145.0	73.5	172.6	0.9	26.4	1.0	0.5	0.5	3.3	9.4	0.4
Non- Autoclaved	117.2	88.5	3.3	209.0	85.7	1060.3	53.5	161.6	81.8	178.6	1.0	33.4	1.2	0.4	0.4	4.2	8.1	0.4

## CHAPTER 3: GENERAL CONCLUSIONS

### 3.1 Conclusions

Runoff from beef cattle feedlots contains manure solids and nutrients and, as such, must be collected and stored until it can be utilized on a growing crop. Because of the low concentration of nutrients in runoff holding pond effluent, its value is relatively low as a crop input. An alternative use for this effluent that adds value to a beef cattle operation could be attractive to producers.

While much research has been focused on algae cultivation from municipal, industrial and agricultural waste streams, comparatively little research has been published describing the feasibility of utilizing the waste streams from beef feedlot operations for this purpose. Digestate from the anaerobic digestion of manure from beef feedlots has the potential to grow *Chlorella sorokiniana* (UTEX 1230), as evidenced by published research, but anaerobic digestion is unlikely to be adopted by most feedlot owners due to significant operational concerns stemming from costs associated with processing the manure before utilization. Likewise, whereas Kobayashi et al. (2013) utilized a hanging bag algae cultivation process, a system utilizing an open raceway or circulated pond design is likely to be more readily adopted by feedlot operators. Initial trials conducted as part of the presented thesis research attempted to build on previous research by Kobayashi et al. (2013) by investigating the ability to cultivate *Chlorella sorokiniana* using anaerobically digested beef manure in a vessel simulating an open raceway design. These initial trials utilized 150-mL glass beakers on an incubating-shaker unit under continuous illumination. Results similar to those reported by Kobayashi et al. (2013)

could not be replicated. In fact, the only growth media that yielded algae growth beyond the first few days was that containing autoclaved digestate, suggesting that competition from other organisms in the digestate was inhibiting algae growth in non-autoclaved treatments. While one alternative may be to digest the manure at a higher temperature to attempt to eliminate microorganisms competing with the algae, an approach where no pre-treatment is utilized for feedlot solids and, instead, beef feedlot runoff holding pond effluent is used to cultivate algae seemed to be a more feasible option for large-scale implementation on a beef feedlot operation. The purpose of the research presented here, therefore, was to identify an appropriate concentration of runoff holding pond effluent alone or supplemented with additional nutrients capable of promoting the growth of *Chlorella sorokiniana*. Because manual enumeration of algae cells is a time-consuming and tedious process, a second objective of this research was to determine the suitability of using a spectrophotometer to determine cell densities in the treatments based upon light absorbance.

Daily manual cell enumeration on a hemocytometer and light absorbance measurements from a spectrophotometer were used to quantify cell growth in treatments containing varying concentrations of beef feedlot runoff holding pond effluent with three treatments supplemented with additional nutrients. Three trials were conducted with each treatment replicated in triplicate during each independent trial.

From this research, the following conclusions resulted:

1. Raw (non-autoclaved) runoff holding pond effluent did not promote or sustain growth of *Chlorella sorokiniana* (UTEX 1230) under the growth conditions

utilized (continuous light, temperature, and agitation of samples in 150-mL beakers).

- a. The only treatment yielding a similar ( $p < 0.05$ ) concentration of algae as the control (Bold's Basal Media) at the conclusion of the 24-day growth period was 60% autoclaved pond effluent diluted with purified water.
  - b. No other treatments were autoclaved and, as such, none yielded sustained algae growth despite supplementation with nutrients (nitrogen and phosphorus).
  - c. Competition with other microorganisms or consumption by other microorganisms is hypothesized as the main inhibitor of algae growth observed in these trials.
2. Light absorbance may provide an acceptable prediction of algal cell density for *Chlorella sorokiniana* (UTEX 1230) grown in beef feedlot holding pond effluent when cell density is greater than 200,000 cells-mL<sup>-1</sup>.
- a. An R-squared ( $r^2$ ) value of 0.75 was calculated for this correlation above 200,000 cells-mL<sup>-1</sup>.
  - b. Suspended solids and non-algae particles obscure light absorbance at low cell densities providing falsely elevated spectrophotometer readings.

Based on these conclusions, it appears unlikely that beef feedlot runoff holding pond effluent is a suitable wastewater stream for cultivating *Chlorella sorokiniana* (UTEX 1230) without some degree of pre-treatment. However, because other researchers

have reported success using digested feedlot manure to cultivate algae, further research may be useful to identify factors capable of inhibiting and/or promoting the growth of algae in runoff holding pond effluent.

### **3.2 Recommendations**

From this research, the following recommendations are offered for consideration when conducting future research of this nature:

1. Analyze multiple beef feedlot runoff holding pond effluent samples to identify microorganisms present that are capable of competing with algae for nutrients and/or are algae consumers.
2. Utilize bubbled oxygen or carbon dioxide in treatments in lieu of agitation, which may not entrain enough air in the liquid to impact algae growth.
3. Utilize a light dark cycle rather than continuous light.
4. Consider pre-treatment methods, such as ultraviolet light, that may be capable of eliminating competing microorganisms from the waste stream prior to its use for cultivating algae.